

Assessing the effects of metal mining effluents on freshwater ecosystems using biofilm as an ecological indicator: comparison between nanofiltration and nanofiltration with electrocoagulation treatment technologies.

Lidia Vendrell-Puigmitja^{a,*}, Meritxell Abril^a, Lorenzo Proia^a, Carmen Espinosa Angona^{a,b}, Marta Ricart^a, Darren L. Oatley-Radcliffe^c, Paul M. Williams^c, Mabrouk Zanain^c & Laia Llenas^a.

^aBETA Tech Center. TECNIO Network, U Science Tech, University of Vic-Central University of Catalonia, C.de la Laura 13, 08500, Vic, Spain.

^bCERM, Center for the Study of Mediterranean Rivers, University of Vic – Central University of Catalonia (UVic-UCC), Manlleu, Spain

^cEnergy Safety Research Institute (ESRI), Swansea University, Bay Campus, Fabian Way, Swansea SA1 8EN, UK.

*Corresponding author. E-mail address: lidia.vendrell@uvic.cat (Lidia Vendrell-Puigmitja)

Abstract

Abandoned mines cause serious environmental damage to their surroundings with considerable impacts on freshwater ecosystems. These impacts occur mainly due to the uncontrolled discharge of polluted effluents, which may contain high concentrations of heavy metals. Currently, no real solution exists for this important environmental problem, leaving a legacy of global pollution. This study aimed to assess the impact of a metal mining effluent from an abandoned mine on freshwater ecosystems, using aquatic biofilms as an ecological indicator. At the same time, the efficiency of different innovative treatment technologies in reducing the ecological impacts caused by mining effluents was evaluated, consisting of nanofiltration and nanofiltration combined with electrocoagulation. To do that, aquatic biofilms obtained from a pristine stream, were exposed, under microcosms conditions, to a metal mining effluent, untreated or treated by the innovative treatment technologies and responses were compared with unexposed biofilm which served as control. The structural and functional responses of the biofilm were measured with throughout time. Biofilms that were exposed to the untreated mining effluent showed significant differences respect to the rest of treatments and the control, particularly exhibiting inhibitory effects on photosynthetic efficiency just after 24 hours of exposure and a progressive shift of the photosynthetic community composition throughout the exposure period. The treatment technologies significantly reduced the ecological impact caused by the metal mining effluent. However, metal bioaccumulation in biofilm revealed a potential long-term impact. These observations evidenced the biofilm as a useful ecological indicator to assess the ecological impact caused by metal

mining effluents on freshwaters and the efficiency of different treatment technologies to reduce it.

Keywords: Aquatic biofilm, Ecological indicator, Metal mining effluents, Treatment technology.

1. Introduction

Mining activities generate large amounts of highly concentrated wastewater due to the contact between water and various types of minerals. The origin of these effluents can be found in the distinct processes undertaken in mining, in addition to drainage from rainfall. Mining effluents can be caused by wash waters, flow process acids, water leaching, flotation and concentrations and from the refining and gas scrubbers (European Commission, 2010). All these processes can generate highly polluted effluents, by carrying soluble substances (as heavy metals) or small particles through soil or rock to the rivers (Kumar, P. 2015).

In some cases, even long after the mining activities have ceased, the emitted metals throughout these effluents continue to persist in the environment (Jain et al., 2016), such as Acid Mine Drainage that occurs when sulphide-bearing material is exposed to oxygen and water (Azapagic, 2004; Johnson and Hallberg, 2005). The environmental problem caused by mining activities is especially critical for abandoned mines, due to the fact that there are no actors in charge of treating these mining effluents. Historically, mine sites were often abandoned without any concerns regarding the potential risks to humans and the surrounding environment, nor with regard to visual impacts, land-scape integration or land-use (European Commission, 2012). Abandoned mines are certain to be of appreciable concern to nearby citizens, as they can affect regional water supplies, contaminate water resources and damage sensitive aquatic habitats (Jain et al., 2016).

Heavy metals may be stored in aquatic ecosystems or seep into the water table, which leads to contamination of underground water courses (Johnson and Hallberg, 2005; Younger et al., 2003). The specific hazards posed by heavy metals harboured in mining effluents to the aquatic environment are highly dependent on the long-term life of the metal, i.e. the metal remains dissolved in water or is adsorbed on suspended solids or sediment, which determines its bioavailability. Bioavailability controls the toxicity of the metal to aquatic communities, which leads to bioaccumulation behaviour and consequently the biomagnification of the metal throughout the trophic web (Younger et al., 2003). Heavy metals dissolved in water are easily absorbed by fish and other aquatic organisms, such as aquatic biofilms which are the primary producers of the aquatic

ecosystems (Solomon, 2008). Metal pollution could lead to a variety of biofilm responses including structural and functional changes with different ecological implications. For example, kinetics of algal growth, photosynthetic activity, chlorophyll-a concentration, nutrient uptake capacity and community composition may be altered in response to chemical stressors harboured in mine effluents (Corcoll et al., 2011). Specifically, metal exposure can produce metabolic and functional alterations on the biofilm and, after long-term exposures, when the accumulation of metals becomes higher, it causes structural changes (Wu, Y., 2016); which indicates the relevance of using a multi-biomarker approach including functional and structural biomarkers (Bonnineau et al., 2010). Within biofilm processes, photosynthesis is critical for algal groups and metal toxicity has been shown to promote its inhibition (Guasch et al., 2002; Serra et al., 2009; Corcoll et al., 2011). The evaluation of ecological impacts caused by metal mining effluents is not trivial (Wu, Y., 2016). The use of biofilm communities as bioindicators can be considered as a good approach of the community ecotoxicology (Sabater et al., 2007). In fact, biofilms are microbial communities made up of bacteria, algae, fungi, and microfauna, located in close physical contact, embedded in a mucopolysaccharide matrix and adhered on any surface in aquatic environments. Biofilms provide a community ecotoxicology approach with higher ecological relevance than using single species and they are a convenient tool to monitor, among others, metal contamination in streams (Leguay et al., 2016; Morin et al., 2012). For these reasons, biofilms are identified within the Water Framework Directive as a biological compartment in European water bodies that should be targeted when aiming to implement optimum ecological integrity (Sabater et al., 2007).

Currently, some technologies exist to treat metal mining effluents in order to minimise the high environmental and ecological impacts that they are causing to water bodies. The conventional active methods for heavy metal removal from wastewaters include chemical precipitation, chemical oxidation, ion exchange, nanofiltration, reverse osmosis, electrocoagulation and electrodialysis (Tripathi and Rawat Ranjan, 2015). Despite the existence of these technologies, the treatment of metal effluents coming from abandoned mines continues to be a major environmental problem, due to the high treatment costs and the lack of establishment actors, responsible to deal with the treatment. In this context, LIFE DEMINE (LIFE16 ENV/ES/000218) is a demonstration project funded by the European Commission that puts in practice, tests and evaluates at pre-industrial scale an innovative treatment process that combines membrane technologies (mainly nanofiltration) and electrocoagulation. Nanofiltration is a relatively new membrane technology and has been in use since the early 1980s. The technology uses the combination of small pore size (nanometre range, MWCO < 2000Da) and

charge to selectively remove divalent cations from solution while allowing monovalent ions to permeate (Mohammad et al., 2015). As a result, this technology has found industrial application in food and beverages, wastewater treatment and drinking water production (Oatley-Radcliffe et al., 2017). Electrocoagulation is essentially chemical precipitation of the metal species by formation of the metal hydroxide, in this case the hydroxyl ions are formed by splitting water at an electrode (Moussa et al., 2017). Any metal that is insoluble in hydroxyl form will then precipitate, thus removing heavy metals but allowing common salts to remain.

Mining effluents are very complex and are composed of several metals. As a result, there are very limited studies available that include representative effects of metal mine effluents on complex biofilm communities. In this study, the efficiency of nanofiltration technology and the combination of nanofiltration + electrocoagulation was evaluated by exposing aquatic biofilms to a metal mining effluent, treated by these technologies or untreated, and structural and functional responses were measured throughout time. It is expected the untreated mine effluent will impact on both the biofilm structure and function, while the treated effluents would have reduced metal content with a consequent lower biological impact for each of the technologies. The maximum reduction should occur when the technologies are combined (Nanofiltration + Electrocoagulation) since this combined treatment was expected to achieve the maximum reduction of the metal content in water.

2. Material and methods

2.1. Mining effluent

This study was conducted in laboratory microcosms using the mining effluent from Frongoch abandoned mine. Frongoch mine is located near the village of Pont-rhyd-y-groes, Ceredigion, and covers approximately 11 hectares. The mine was exploited for lead (Pb) and zinc (Zn) extraction for almost 200 years until the early 1900s. The Frongoch mining effluent discharges into the Afon Ysywyth catchment. The specific metal and nutrient composition of the mining effluent off Frongoch mine used in this study was 6.91 mg L^{-1} of Pb, 0.55 mg L^{-1} of cadmium (Cd) and 76.07 mg L^{-1} Zn, 0.79 mg L^{-1} of dissolved inorganic nitrogen (DIN), 0.03 mg L^{-1} of phosphate (PO_4^{3-}), 13.56 mg L^{-1} of sulphate (SO_4^{2-}), pH of 6.91 and alkalinity of 12.13 mg L^{-1} . The average annual rainfall in the Afon Ysywyth catchment is almost 2000 mm per year (1961-1990 average) (Bearcock et al., 2010), and the flow rate of the river which receive the mining effluent has been measured on average at $100 \text{ m}^3 \text{ h}^{-1}$ (Edwards and Williams, 2016).

The Frongoch mining effluent has been treated at bench scale using the treatment technologies developed in LIFE DEMINE. This study has been performed using: an unpolluted stream water as control (C), (ii) the treated effluent using only the nanofiltration step (NF), (iii) the treated effluent using nanofiltration combined with electrocoagulation (NF+E) and (iv) the untreated (U) effluent from Frongoch mine.

2.2. Experimental design

Twelve microcosms consisting of glass aquariums (length × width × height 26 × 15 × 17 cm) were used to assess the biofilm response under untreated and treated mining effluents (Figure 1). Each microcosm contained 15 previously scraped and autoclaved stream cobbles that were used as substrata for natural biofilm colonization, and each microcosm was filled with 3-L of artificial water. Artificial water was prepared to mimic a pristine stream as described in Ylla et al. (2009). The artificial water was obtained by dissolving pure salts in distilled water creating a chemical composition of 0.75 mg L⁻¹ N, 0.10 mg L⁻¹ P, 15.83 mg L⁻¹ Na⁺, 8.17 mg L⁻¹ Ca²⁺, 0.52 mg L⁻¹ K⁺, 0.19 mg L⁻¹ Mg⁺², 8.89 mg L⁻¹ SO₄²⁻, 10.71 mg L⁻¹ SiO₃²⁻, 14.94 mg L⁻¹ Cl²⁻ and 14.52 mg L⁻¹ HCO₃⁻. Water was recirculated in each aquarium by a submersible pump (EDEN 105, Eden Water Paradise, Italy). The water in the aquaria was completely renewed every 3-4 days to avoid nutrient depletion. The temperature and the photoperiod were set at 18°C and 12 h light: 12 h dark using LEDs (LENB 135-lm, LENB/14.97/11.98). During the experiment, the irradiance reaching the substrata was 12 μmol photons m⁻² s⁻¹.

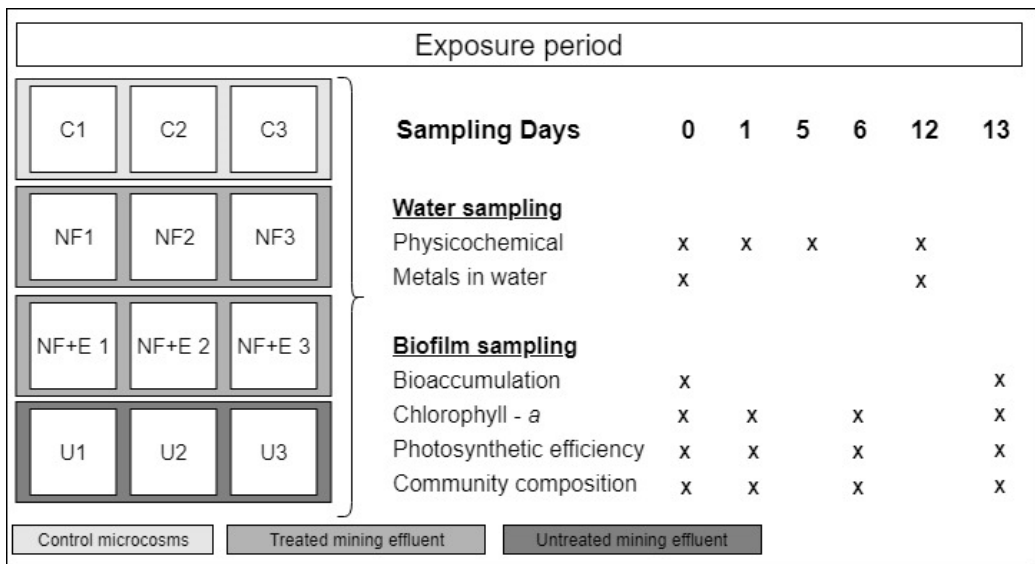


Figure 1. Experimental design and sampling strategy during the exposure period. C = control, U = untreated mining effluent, NF = nanofiltration and NF+E = combined treatments.

To promote biofilm colonisation, 15 ml of natural biofilm suspension obtained by scraping cobbles collected from Viladrau pristine and forested stream (Natural Park of Montseny,

Spain) were inoculated in each microcosm. This inoculum was added at the beginning of the experiment to favour biofilm settlement, as well as after each water renewal during the colonization period, which lasted for three weeks.

After the colonization, the exposure period started with the addition of water from the mining effluents. Four different treatments were tested to compare biofilm responses with means of a control (C) in which the conditions remained unvaried. As previously explained (in section 2.1), the evaluated treatments were: untreated mining effluent (U) and mining effluent treated by: nanofiltration (NF) and nanofiltration combined with electrocoagulation (NF+E). The exposure was set to mimic the real dilution conditions in Afon Ysywyth river where a mining effluent of $4.3 \text{ m}^3 \text{ h}^{-1}$ reaches the Afon Ysywyth river of $100 \text{ m}^3 \text{ h}^{-1}$ (Edwards and Williams, 2016). To achieve these conditions, 0.2 L of each effluent was added to 2.8 L of artificial water in the respective microcosms and each experiment was performed in triplicate. During the exposure period, which lasted for 13 days, each effluent was added to the respective microcosms at each water renewal every 3 to 4 days.

3. Sample analysis

3.1. Physico-chemical water conditions

During the experiment, light intensity and water temperature were continuously monitored with HOBO Pendant (Onset Computer Corporation, Bourne, USA) on five randomly selected microcosms, including one per each treatment. At each water renewal, the position of the HOBO was changed (within replicates of the same treatment) in order to ensure the collection of data from all the microcosms at the end of the experiment.

Dissolved oxygen concentration and saturation (YSI professional plus, YSI Incorporated, USA), pH (G-PH7-2 portable pH meter XS PH7 + DHS) and conductivity (G-COND7-2 conductivity-meter portable XS COND 7+) were measured directly in the microcosms with sensor probes. Triplicate water samples for each treatment were collected and filtered through a $1.2 \mu\text{m}$ pore diameter glass microfiber filter (Prat Dumas Filter Paper, Couze-St-Front, France) to analyse NO_2^- (APHA et al., 1992a), NO_3^- (APHA et al., 1992b), NH_4^+ (Reardon et al., 1966) and PO_4^{3-} (Murphy and Riley, 1962). In addition, a water sample for each microcosm was collected and filtered through a $1.2 \mu\text{m}$ pore diameter glass microfiber filter (Prat Dumas Filter Paper, Couze-St-Front, France) to analyse metal concentrations in water before (t_0) and at the end of the exposure period (t_{12}), using a Microwave Plasma Atomic Emission Spectroscopy (MP-AES model 4200,

Agilent technologies, UK). All water samples were frozen at -20°C after sampling until the analysis.

3.2. Biofilm sampling

Biofilms were sampled before (t₀) and after 1, 6 and 13 days of the effluent's addition, always during the day following the water renewal (Figure 1). At each sampling day, three random cobbles from each microcosm were scrapped using a toothbrush and were suspended in 10 mL of the corresponding microcosm water. The area of stones was measured placing silver paper on the top of the stone and delineating its surface. Biofilm photosynthetic activity and the phototrophic community composition were measured immediately after collection, whereas samples for AFDM and chlorophyll-a concentration on the biofilm were stored at -20°C until analysis. Samples for the determination of metal bioaccumulation in biofilm were taken before the exposure (t₀) and at the end of this period (t₁₃).

3.2.1. Metal bioaccumulation in biofilm

In order to measure the metal bioaccumulation in biofilm, dried biofilm samples were lyophilized and weighed (g) to determine the dry weight (DW) (Corcoll et al., 2012). Then, 200mg of DW were digested with 4mL of concentrated HNO₃ (65%, suprapure) and 1mL of H₂O₂ (31%, suprapure). After dilution with MiliQ water, water samples were acidified (1% nitric acid suprapure) and stored at 4 °C. Dissolved metals of the digested samples were analysed by ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE). Metal bioaccumulation was expressed as dissolved metal contents per biofilm dry weight (μg g⁻¹ DW).

3.2.2. Chlorophyll-a concentration

Chlorophyll-a concentration on the biofilm was measured after extraction with 90% acetone for 24 hours in the dark at 4 °C. Extracts were filtered through 47 mm diameter and 1.2 μm pore filter (A0258855 Prat Dumas), and chlorophyll-a concentration was further determined by spectrophotometric measurements (A3-50S17R-U R01101900571) following the method described in Jeffrey and Humphrey (1975). The chlorophyll-a physiological state was determined by calculating the Margalef index (Margalef, 1983).

3.2.3. Photosynthetic activity and community composition

The chlorophyll-a fluorescence emission of the biofilms was measured with a Mini PAM (Pulse Amplitude Modulated) chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich,

Germany), which uses light-emitting diodes that excite chlorophyll. The measurements of the photosynthetic activity were carried out by placing the sensor directly on three different zones of each colonized cobbles. BenthosTorch (bbe Moldaenke, Schwentineta, DK) was used for a real-time measuring of the main photosynthetic groups' densities (diatoms, cyanobacteria and green algae) of biofilm communities. Measurements were done covering the colonized cobble with the probe, which uses the in vivo fluorescence of algal cells to determine the concentration of the main groups (Echenique-Subiabre et al., 2016).

4. Data Analysis

Physico-chemical data, photosynthetic efficiency, metal concentration in water, metal bioaccumulation and chlorophyll-a concentration in biofilm were evaluated using one-way repeated measures analysis of variance (ANOVA) in SPSS Statistics version 21, with treatment (C, NF, NF+E and U) as factor and sampling date (time) as repeated measure. For physico-chemical data, a separate ANOVA was done for the colonisation and exposure period. Effects were analysed *post hoc* with a Tukey-b test when one-way ANOVA revealed significant differences among treatments. One-way ANOSIM tests (using Bray-Curtis similarity coefficients) were performed on relative abundances of the biofilm community composition at each sampling time with Bonferroni correction in Past3 version 3.23. Pearson correlation analysis was performed to explore the relationship between the community composition and the bioaccumulated metals in biofilm. A principal component analysis (PCA) using R Studio software (version 3.6.0) was performed to visualize the differences among treatments at the end of the exposure period (t13) based on the photosynthetic efficiency, metal bioaccumulation and the relative abundance of algal groups (cyanobacteria, green algae and diatoms) of the biofilm.. Statistical significance was set at $p < 0.05$ for all the performed tests.

5. Results

5.1. Physico-chemical water parameters

During the colonisation period, the physico-chemical conditions did not show significant differences among microcosms. During the exposure period, dissolved oxygen, water temperature and pH remained stable with no significant deviations (Table 1). Nutrient concentrations showed similar behaviour in all microcosms and decreased between water renewals: PO_4^{3-} from 134 (± 12) to 62 (± 11) $\mu\text{g L}^{-1}$ and NO_3^- from 810 (± 20) to 450 (± 18) $\mu\text{g L}^{-1}$, the N/P ratio range during the whole experiment was between 14 and 18. The dissolved metal concentrations analysis showed that Zn was the most abundant metal during the exposure period in all the microcosms exposed to the effluents, with the

highest concentration found in the microcosms exposed to the U compared to the treated ones and the C (one-way ANOVA $F=23.06$ $p<0.05$; Tukey test, $p<0.05$) (Table 1). By contrast, Pb and Cd concentrations in water were below the detection limit in all microcosms during the experiment (detection limit 0.01 mg L^{-1}).

Table 1. Physico-chemical conditions of the microcosms water during the colonisation (n=9) and exposure period (n=4) (mean \pm SD). Zinc (Zn) concentration is expressed as the average between t0 and t12 (n=2). C= control, U=mining effluent, NF=nanofiltration and NF+E=combined treatments. n.a = data not available, n.d =not detected.

	Colonisation period (n=9)	Exposure period (n=4)			
		C	NF	NF+E	U
Temperature ($^{\circ}\text{C}$)	21.85 (± 0.44)	22.23 (± 0.03)	21.79 (± 0.03)	20.10 (± 0.04)	21.53 (± 0.03)
pH	7.93 (± 0.10)	7.88 (± 0.15)	8.01 (± 0.06)	8.05 (± 0.07)	7.76 (± 0.06)
O ₂ (mg L^{-1})	7.76 (± 0.14)	7.26 (± 0.15)	7.66 (± 0.09)	6.97 (± 0.12)	7.34 (± 0.10)
Conductivity ($\mu\text{S cm}^{-1}$)	147.01 (± 2.21)	173.57 (± 9.15)	151.68 (± 3.26)	149.49 (± 3.78)	174.96 (± 2.72)
Zn (mg L^{-1})	n.a	n.d	0.36 (± 0.03)	0.15 (± 0.04)	4.25 (± 1.15)

5.2. Biofilm parameters

5.2.1. Chlorophyll-a concentration

Chlorophyll-a concentration in the biofilm decreased progressively during the exposure period in all treatments without significant differences among them in any of the sampling dates. Just before the starting of the exposure period (t0), the chlorophyll-a in all treatments was on average $3.5 (\pm 1.9) \mu\text{g cm}^{-2}$ (n=36) while at the end of the exposure period (t13) it was $1.5 (\pm 0.8) \mu\text{g cm}^{-2}$ (n=36).

5.2.2. Photosynthetic efficiency (Yeff)

The biofilm photosynthetic efficiency (Yeff) during the exposure period tended to increase over time in all treatments except for the case of U-exposed biofilms (Figure 2) which presented some significantly lower Yeff values than the rest of the treatments and C (ANOVA repeated measures $F=48.88$, $p<0.05$; Tukey test, $p<0.05$). In particular, the Yeff decreased drastically for the U effluent, since the first day of exposure (t1), when significant differences appeared between this treatment and the others (one-way ANOVA $F=4.89$, $p<0.05$; Tukey test, $p<0.05$). On the other hand, NF+E treatment showed higher Yeff values than C and NF at the end of the exposure period (t13) (one-way ANOVA $F=30.65$, $p<0.05$; Tukey test, $p<0.05$).

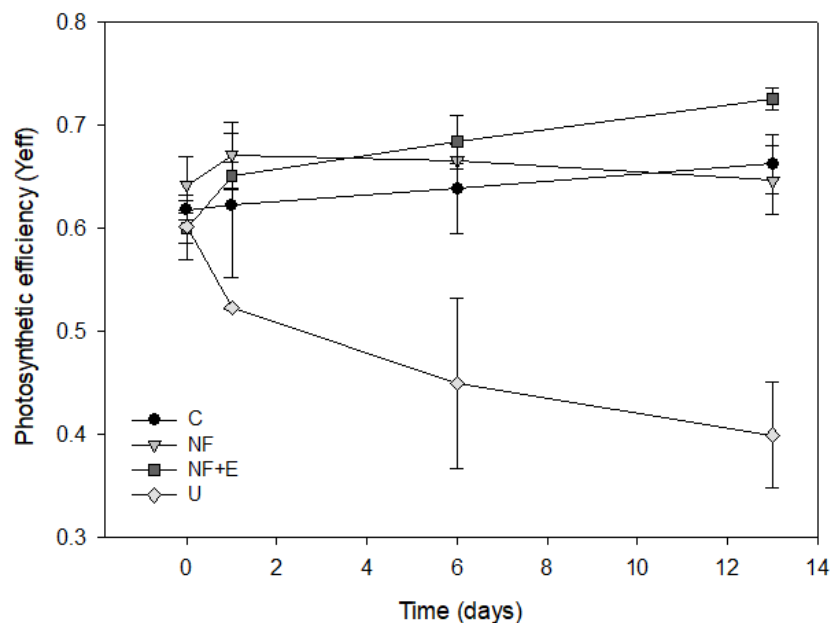


Figure 2: Photosynthetic efficiency (Y_{eff}) of the biofilm on each treatment during the exposure period, mean \pm SD ($n=3$). C= control, U= untreated mining effluent, NF= nanofiltration and NF+E= combined treatments.

5.2.3. Photosynthetic community composition

Just before the beginning of the exposure period (t_0), all microcosms showed similar photosynthetic community composition (Figure 3), dominated by diatoms ($73\pm4\%$) followed by cyanobacteria ($25\pm4\%$) and a negligible presence of green algae ($1\pm1\%$) (ANOSIM, $R=0.02$, $p=0.27$). By contrast, a clear shift of the community composition was observed for the U effluent samples, just after 6 days of exposure (t_6) (Figure 3), characterized by a significant increase of the relative abundance of green algae in the biofilm community compared to the other treatments (one-way ANOSIM, $R=0.04$, $p<0.05$). This trend continued until the end of the exposure period (t_{13}) when green algae dominated ($73\pm3\%$) the biofilm community in the U system, significantly differing from the other treatments (ANOSIM, $R=0.08$, $p<0.05$; (Figure 3). The rest of treatments (NF and NF+E) did not present changes in the biofilm photosynthetic community composition from t_0 until t_{13} , following the same trend as C (Figure 3).

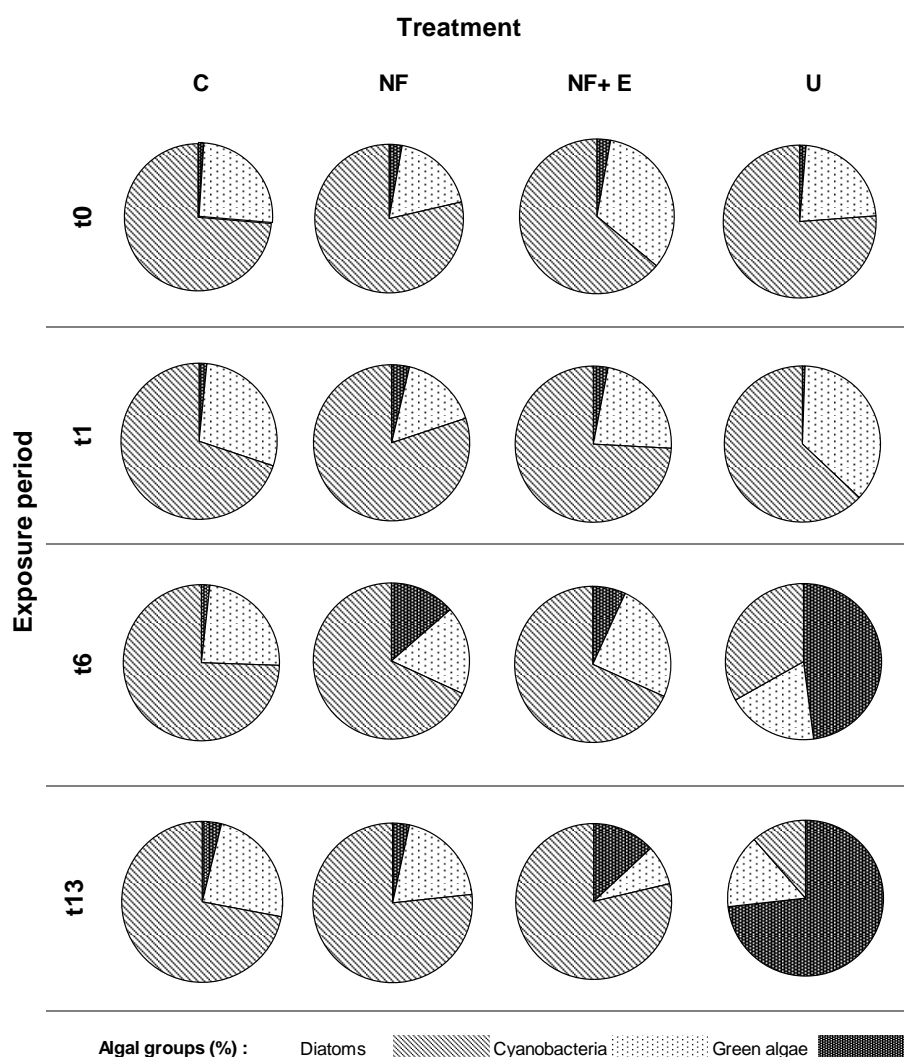


Figure 3: Relative abundance (%) of each algal group conforming the photosynthetic community composition of the biofilm on each treatment along the exposure period. C= control, U= untreated mining effluent, NF= nanofiltration and NF+E= combined treatments. The results present the mean values of three replicates of each microcosm at each sampling date.

5.2.4. Metal bioaccumulation

Just before the exposure (t0), the metal concentrations in the biofilms were similar among the microcosms. At the end of the exposure period (t13), significant differences were found in the Zn (one-way ANOVA, $F=23.5$, $p<0.05$), Pb (one-way ANOVA, $F=152.7$, $p<0.05$) and Cd (one-way ANOVA, $F=50.7$, $p<0.05$) metal content accumulated in the biofilms depending on the treatment. These differences were especially evident between the biofilm exposed to the U effluent and the C (Figure 4). Biofilms that were exposed to the U effluent contained 18-fold more Zn, 46-fold more Pb and 25-fold more Cd than C biofilms. On the other hand, the biofilm that was exposed to the effluent treated by NF+E presented 3-fold more Zn bioaccumulation with respect to the C. In contrast, biofilms

exposed to NF treated effluent did not present significant differences with the C for any of the metals measured (Figure 4).

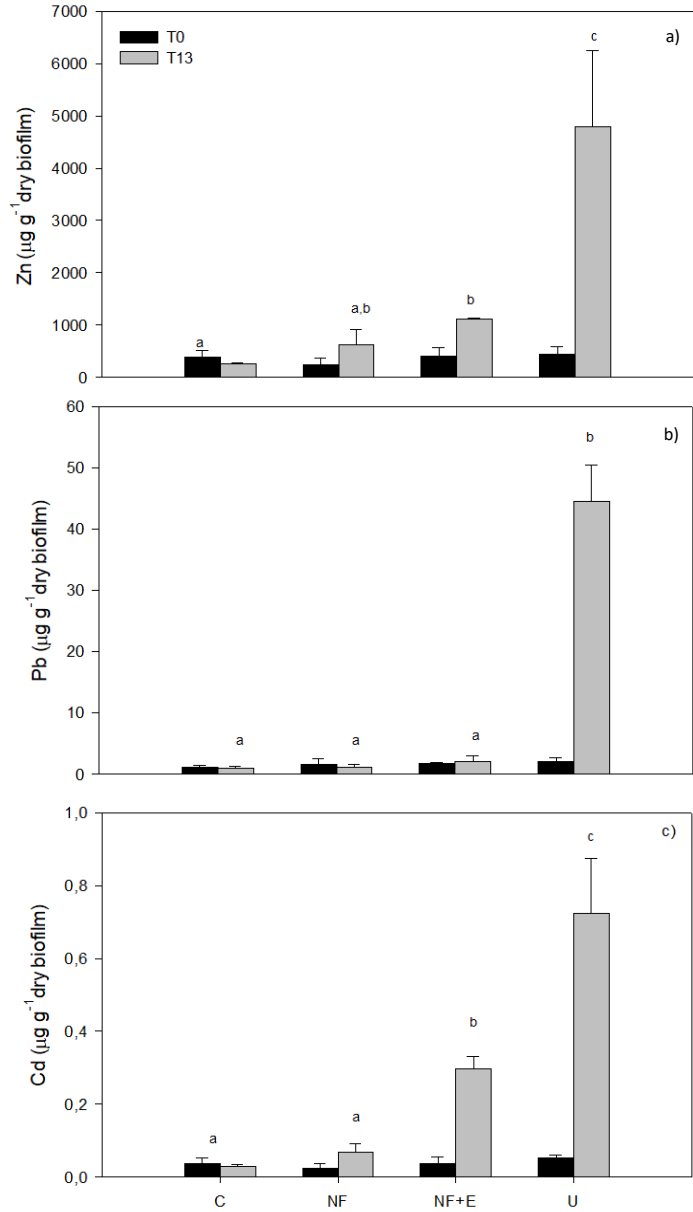


Figure 4. Metal bioaccumulation in biofilms just before the exposure period (t0) and after 13 days of exposure (t13) to the different treatments. C= control, U= untreated mining effluent, NF= nanofiltration and NF+E= combined treatment. Mean \pm SD ($n = 3$). The letters indicate significant differences ($p < 0.05$) between treatments after one-way ANOVA and Tukey's HSD test. a) indicates the Zn bioaccumulation. b) is for Pb bioaccumulation and c) Cd bioaccumulation in biofilm.

A significant positive correlation was found between green algal biomass, based on the main photosynthetic groups densities, and Zn bioaccumulation (Pearson's correlation $r=0.83$, $p<0.01$) on the biofilm at the end of the exposure period (t13), which increased in a significant linear relationship (linear regression, $R^2=0.69$, $p<0.01$). By contrast, a negative correlation between diatoms biomass and Zn bioaccumulation was found

(Pearson's correlation, $r=-0.81$, $p<0.01$), which decreased in a significant linear relationship (linear regression, $R^2=0.66$, $p<0.01$).

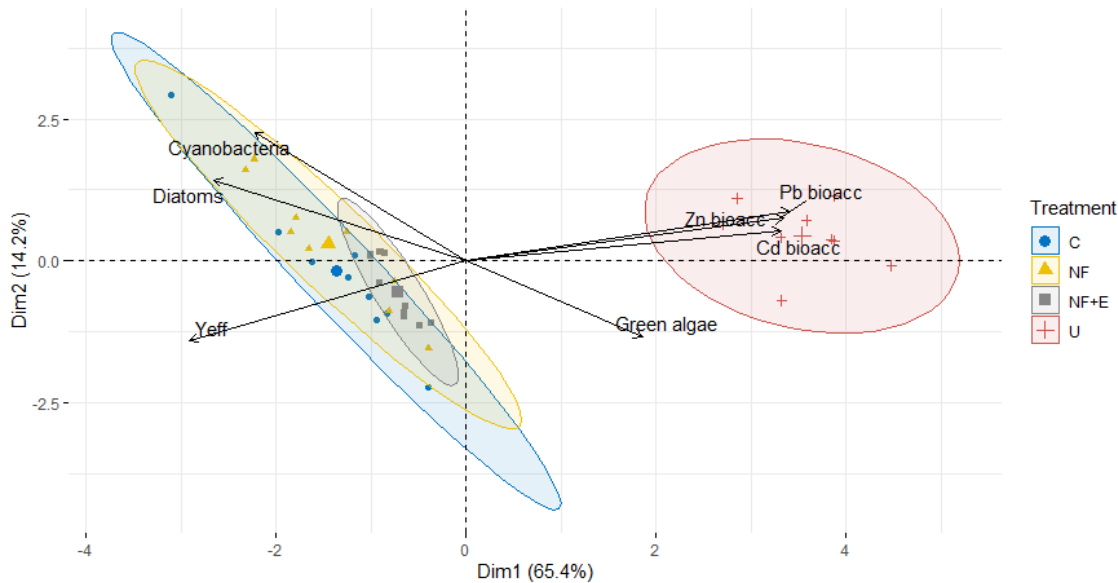


Figure 5: Biplot of the principal component analysis (PCA), with data points identified by treatment. Vectors plotted indicate the correlation scores between the community composition, metals bioaccumulation and photosynthetic efficiency at the end of the experiment (t13). The ellipses indicate 95% confidence around their centroids for treatment.

A principal component analysis (PCA) was used to visualize the difference among treatments at the end of the exposure period based on the community composition metal bioaccumulation and photosynthetic efficiency of the biofilm. The first axis of the PCA (explaining 65.4% of the variance) was linked to the pollution gradient, with higher values of metals bioaccumulated and the dominance of green algae in the U treatment. By contrast, C and treatments (NF and NF + E) appeared at the opposite side of this axis, indicating similar characteristics under a less impacted scenario. The data were plotted within the correlation circles and differentiated two main trends, the U treatment from the C, NF and NF+E.

6. Discussion

The primary aim of this work was to investigate the impacts of an abandoned metal mine effluent on freshwater ecosystems and the efficiency of different treatment technologies in reducing these impacts, using the aquatic biofilm as ecological indicator. The experimental setup allowed to simulate the chemical conditions generated by the entrance of a real metal mining effluent to a stream in order to assess the impact caused

on natural aquatic biofilm, before and after the effluent treatment with two different technologies. The metal mining effluent used in this study was characterised by a high Zn concentration, thus the U effluent presented the highest Zn concentration in water ($4.25 \pm 1.15 \text{ mg Zn L}^{-1}$), which exceeded both European and US legislation limits, according to the Water Framework Directive (WFD) (Directive 2000/60/EC, 2000) and US Environmental Protection Agency (US EPA, 2014) respectively.

In this study, a variety of biofilm community alterations has been observed, including changes in the photosynthetic efficiency, community composition and metal bioaccumulation mainly caused by the exposure to U mine effluent waters. Photosynthetic efficiency, expressed as quantum yield is a sensitive indicator of metal toxicity to biofilms (Miller et al., 2017). In this study, as Tlili et al. (2011) and Bonet et al. (2014) demonstrated in similar studies, a drastical decrease in the photosynthetic efficiency was observed in biofilms under the untreated mining (U) effluent compared to the C just after 24h of exposure. The observed decrease of the photosynthetic efficiency indicated the damage produced by the metal exposure on the photosynthetic apparatus (Leal-Alvarado et al., 2016; Miller et al., 2017). In this regard, other studies evidenced that Zn concentrations of $450 \text{ } \mu\text{g Zn L}^{-1}$ to 40 mg Zn L^{-1} in water, similar to those generated by the exposure to U mining effluent in our study, are enough to affect biofilm photosynthesis after short-term exposure (Bonet et al., 2013; Blanck et al., 2003). This decrease of the photosynthetic efficiency could be explained by the mode of action of Zn potentially targeting several photosynthetic processes of algae and cyanobacteria (Corcoll et al., 2012). For example, the in vivo substitution of magnesium, which is the central atom of chlorophyll-a, by heavy metals (such as Zn), could lead to a breakdown in photosynthesis (Corcoll et al., 2011).

In addition to the observed decrease in the photosynthetic efficiency, biofilms exposed to the U mining effluent also experimented a marked shift in the photosynthetic community composition after longer-term exposure (t13). Indeed, the relative abundance of green algae in these biofilms increased throughout the exposure period compared to the control, where diatoms dominated during the whole experiment. Similar results were reported by Corcoll et al. (2011) and Ivorra et al. (2000) that found that green algae were favoured after several weeks of exposure to Zn in microcosms compared to diatoms. In this sense, significant higher abundances of green algae have been reported at different confluences of mining effluents with stream waters in natural environments (Das and Ramanujam, 2011). Different mechanisms could enable green algae to tolerate chemical stress caused by heavy metal concentrations such as a decrease in the number of

binding sites at the cell surface, internal detoxifying mechanisms (Gold et al., 2003) or additional enzymatic activity provided by metals (Pawlik-Skowronska 2003). These mechanisms were described by Corcoll et al. (2012) and are related to Zn toxicity that may enhance the synthesis of antioxidants causing the activation of the xanthophyll cycle of green algae as protective mechanisms to avoid Zn toxicity. By contrast, we observed a clear decrease in the relative abundance of diatoms in the biofilm under the effect of the U mining effluent at the end of the exposure period. Higher abundances of deformed diatoms have been reported in zinc polluted sites, which are indicative of toxic stress (Morin et al., 2014).

The functions of biofilms can be recovered after long term of exposure because the tolerant species ensure these functions by species replacement (Guasch et al. 2010, Corcoll et al. 2012, Tlili et al. 2010 and Barranguet et al. 2000). However, in the present study the biofilm functioning has not been recovered at the end of the exposure period. It is well known that high concentrations of Zn can inhibit the photosynthetic activity by blocking electron transfer at PSI and PSII levels, which stops oxygen production and CO₂ fixation (Ivorra et al. 1999).

Biofilms exposed to the U effluent indicated an ecological impact by showing inhibitory effects on photosynthetic efficiency just after 24 hours of exposure and a progressive shift of the photosynthetic community composition throughout the exposure period. This shift coincided with an increase of Zn, Pb and Cd bioaccumulation in these biofilms exposed to the U effluent compared to the C communities. Significant relationships between metal bioaccumulation in biofilms and dissolved metal concentration in water column have been found (Leguay et al., 2016), suggesting that the metal concentration in water explains a large amount of the variability in metal concentration inside biofilms. Biofilms have a large number of metal binding sites located in either mucopolysaccharide at the surface of cells or in the organic particles trapped by the biofilm. These substances can play an important role in the sorption of dissolved metals from water column on biofilms (Bere et al., 2012; Duong et al., 2008, 2010). The bioaccumulation of trace metals by algal cells is well known and generally increase with exposure time (Collard and Matagne, 1994). Green algae have the capacity to concentrate inorganic ions to amounts several thousand folds greater than in external dilute solutions by a variety of biological, chemical and physical mechanisms involving adsorption, precipitation and metabolism-dependent processes that operate simultaneously or in sequence (Bere et al., 2012). Metals become toxic for algae when intracellular metal is present at high concentrations and exerts a negative influence on the biochemical mechanisms occurring within the cells (Corcoll et al., 2012). The presence of metal ions can affect

many aspects of biofilm communities including biomass, metabolic activity (e.g., enzyme activity, photosynthesis), and extracellular polymeric substances (EPS) productivity (Tang et al., 2017). In this regard, Zhu et al (2019) demonstrated that EPS productivity play the most important role in the community composition shift due to metal ion presence. EPS have many functional groups including carboxylates, hydroxyls, phosphates and sulphates (Sheng et al., 2010), which can change the size, distribution and surface properties of nanoparticles and thereby stabilize its dispersion or induce their aggregation (Quigg et al., 2013). As a result, EPS can be considered a protective barrier of microbial cells in periphytic biofilms from the exposure of nanoparticles (Joshi et al., 2012). However, the EPS production and composition changes of periphytic biofilms under nanoparticles exposure have been rarely reported (Liu et al., 2019).

Regarding the treatment technologies, biofilms exposed to the treated mining effluents showed similar responses than C biofilm, indicating the reduction of the ecological impact caused by the U, and therefore, the efficiency of the treatment technologies tested. In this regard, biofilms exposed to NF and NF+E did not show any significant decrease in the photosynthetic efficiency compared to the control during the exposure period. However, the biofilms exposed to NF+E treated effluent presented an increase of the photosynthetic efficiency above the control at the end of the experiment (t13). Ivorra et al. (2002) suggested that another adaptive response of individual algal species is the maintenance of a high photosynthetic efficiency under Zn stress. Indeed, the biofilm exposed to NF+E treatment presented 3-fold more Zn bioaccumulation with respect to the C. It should be noted that the Zn content of the effluent obtained by both treatments were still exceed the EU standards (0.12 mg L^{-1} , EC 2000). The main reason for that is because NF and E technologies were combined with the final aim to increase the water recovery rather than improving the NF permeate quality. In that sense, the E step was treating the NF concentrate. After the E step, a sludge with a high metal concentration and a clean water effluent was obtained. The clean water effluent after the E was mixed with the NF permeate, but the results obtained showed that the E process was not very efficient and due to the high amount of metals present in the NF concentrate, the mixture of the two clean water effluents (NF+E) resulted in a more polluted effluent than the NF.

The results obtained in this study contribute to a better understanding in the evaluation of the efficiency of treatment technologies that permit to reduce the ecological impact to the natural stream of a metal effluent, and additionally to the greater understanding of the response of fluvial biofilms to a metal mining effluent exposure. The technologies proposed to treat the metal mining effluents has proved to be a viable and valid option since the technology significantly reduced the amounts of metals present in the mine

effluent. Due to this reduction, the biofilm functioning (photosynthetic efficiency) and structure (community composition) in biofilms exposed to the treated effluents did not show substantial differences respect to the C. At the same time, metal bioaccumulation in biofilm decreased drastically in biofilms exposed to these treated effluents compared to the U one, being equal to the C, in the case of NF. However, metal bioaccumulation on biofilm under the NF + E treatment was higher than the C, indicating that the mining effluent treated by these this technology could still cause certain environmental impact on the ecosystem, being evident only in the long-term.

7. Conclusions

Aquatic biofilms revealed that the tested treatment technologies offer an effective and viable solution to treat these metal effluents from abandoned mines. These technologies have the potential to significantly reduce the global environmental and ecological impact of mining operations and highlights the need to treat these highly polluting effluents. This study also evidences the ecological impact caused by the high concentrations of metals such as Zn, Cd and Pb in aquatic biofilms and the overall ecosystem as a result of abandoned metal mines. The work demonstrates the ability of biofilms to act as biological indicators of metal pollution due to their sensitivity and ability to accumulate metals from low concentrations in water, even below detection limits. In this regard, an effect in both functional and structural capacities as a shift of the phototrophic community composition and the decrease on the photosynthetic efficiency was observed. Therefore, the need to treat this metal effluent was highlighted, since the negative impact they generate on aquatic ecosystems was demonstrated. The development of this study has not only been useful to properly evaluate the negative ecological impacts caused by the metal mining effluents, but it has also been highly important to help in the improvement of the treatment system. Thanks to the results obtained, it has been seen that the combination of the NF and E technologies in the way initially planned (NF + E) was not as efficient as expected, indicating that combining the two technologies in a different way could be tested as an alternative to improve the efficiency of the technology in reducing the environmental and ecological impacts.

Funding

This work was supported by the European Commission LIFE program throughout the LIFE DEMINE project (LIFE16 ENV/ES/000218).

Bibliography

APHA (American Public Health Association), 1992a. Method 4500 - NO₂⁻. Nitrogen

521 (Nitrite)*. Standard Methods for the Examination of Water and Wastewater, 18th ed.
 522 Washington, D.C.

523 APHA (American Public Health Association), 1992b. Method 4500-NO₃⁻. Nitrogen
 524 (Nitrate)*. Standard Methods for the Examination of Water and Wastewater, 18th ed.
 525 Washington, D.C.

526 Azapagic, A., 2004. Developing a framework for sustainable development indicators for
 527 the mining and minerals industry. *J. Clean. Prod.* 12, 639–662.
 528 [https://doi.org/10.1016/S0959-6526\(03\)00075-1](https://doi.org/10.1016/S0959-6526(03)00075-1)

529 Barranguet, C., Charantoni, E., Plans, M., Admiraal, W., 2000. Short-term response of
 530 monospecific and natural algal biofilms to copper exposure. *Eur. J. Phycol.* 35, 397–
 531 406. <https://doi.org/10.1080/09670260010001736001>

532 Bearcock, J., Palumbo-Roe, B., Banks, V., Klinck, B., 2010. The hydrochemistry of
 533 Frongoch Mine, Mid Wales. *Br. Geol. Surv.* 1–44.

534 Bere, T., Chia, M.A., Tundisi, J.G., 2012. Effects of Cr III and Pb on the bioaccumulation
 535 and toxicity of Cd in tropical periphyton communities: Implications of pulsed metal
 536 exposures. *Environ Pollut* 163, 184–191, doi:10.1016/j.envpol.2011.12.028.

537 Blanck, H., Admiraal, W., Cleven, R.F.M.J., Guasch, H., Van den Hoop, M.A.G.T., Ivorra,
 538 N., Nyström, B., Paulsson, M., Petterson, R.P., Sabater, S., Tubbing, G.M.J., 2003.
 539 Variability in zinc tolerance, measured as incorporation of radio-labeled carbon
 540 dioxide and thymidine, in periphyton communities sampled from 15 European river
 541 stretches. *Arch. Environ. Contam. Toxicol.* 44, 17–29.
 542 <https://doi.org/10.1007/s00244-002-1258-4>

543 Bonet, B., Corcoll, N., Acuña, V., Sigg, L., Behra, R., Guasch, H., 2013. Seasonal
 544 changes in antioxidant 541 enzyme activities of freshwater biofilms in a metal
 545 polluted Mediterranean stream. *STOTEN.* 444, 60–72.
 546 <https://doi.org/10.1016/j.scitotenv.2012.11.036>.

547 Bonet, B., Corcoll, N., Tlili, A., Morin, S., Guasch, H., 2014. Antioxidant enzyme activities
 548 in biofilms as biomarker of Zn pollution in a natural system: An active bio-monitoring
 549 study. *Ecotoxicology and Environmental Safety*, Volume 103, Pages 82-90, ISSN
 550 0147-6513, <https://doi.org/10.1016/j.ecoenv.2013.11.007>.

551 Bonnineau, C., Guasch, H., Proia, L., Ricart, M., Geiszinger, A., Romaní, A. M., &
 552 Sabater, S. (2010). Fluvial biofilms: a pertinent tool to assess β -blockers toxicity.
 553 *Aquatic Toxicology*, 96(3), 225-233.

554 Burello, E., & Worth, A. P. (2011). A theoretical framework for predicting the oxidative
555 stress potential of oxide nanoparticles. *Nanotoxicology*, 5(2), 228-235.

556 Chen, G. (2004). Electrochemical technologies in wastewater treatment. *Separation and*
557 *purification Technology*, 38(1), 11-41.

558 Collard, J., Matagne, R.F., 1994. Cd 2+ resistance in wild-type and mutant strains of
559 *Chlamydomonas Reinhardtii* 34, 235–244.

560 Corcoll, N., Bonet, B., Leira, M., Guasch, H., 2011. Chl-a fluorescence parameters as
561 biomarkers of metal toxicity in fluvial biofilms: an experimental study. *Hydrobiologia*,
562 673, pp. 119-136, 10.1007/s10750-011-0763-8.

563 Corcoll, N., Bonet, B., Morin, S., Tlili, A., Leira, M., Guasch, H., 2012. The effect of metals
564 on photosynthesis processes and diatom metrics of biofilm from a metal-
565 contaminated river: A translocation experiment. *Ecol. Indic.* 18, 620–631.
566 <https://doi.org/10.1016/j.ecolind.2012.01.026>

567 Das, M., Ramanujam, P., 2011. Metal content in water and in algae in coal mine impacted
568 streams. *Int. J. Bot.* 7 (2), 170–176.

569 Directive 2000/60/EC, 2000. Establishing a framework for Community action in the field
570 of water policy (2000). *Official Journal L327*, p. 1-73.

571 Duong, T.T., Morin, S., Coste, M., Herlory, O., Feurtet-Mazel, A., Boudou, A., 2010.
572 Experimental toxicity and bioaccumulation of cadmium in freshwater periphytic
573 diatoms in relation with biofilm maturity. *Sci. Total Environ.* 408, 552–562.
574 <https://doi.org/10.1016/j.scitotenv.2009.10.015>

575 Duong, T.T., Morin, S., Herlory, O., Feurtet-Mazel, A., Coste, M., Boudou, A., 2008.
576 Seasonal effects of cadmium accumulation in periphytic diatom communities of
577 freshwater biofilms. *Aquat. Toxicol.* 90, 19–28.
578 <https://doi.org/10.1016/j.aquatox.2008.07.012>

579 Edwards, P., Williams, T., 2016. Abandoned Mine Case Study: Cwmystwyth Lead Mine
580 2015–2016.

581 EC. Directive 2000/60/EC of the European Parliament and of the Council establishing a
582 framework for the Community action in the field of water policy. *Official Journal L*
583 *327*, P. 0001-0073 (22/12/2000); 2000.

584 Echenique-Subiabre, I., Dalle, C., Duval, C., Heath, M. W., Couté, A., Wood, S. A., ... &
585 Qublier, C. (2016). Application of a spectrofluorimetric tool (bbe BenthosTorch) for

586 monitoring potentially toxic benthic cyanobacteria in rivers. Water research, 101,
587 341-350.

588 European Commission (2012), Supporting document on closure methodologies for closed
589 and abandoned mining waste facilities. Annex 3.
590 [https://ec.europa.eu/environment/waste/mining/pdf/Annex3_closure_rehabilitation](https://ec.europa.eu/environment/waste/mining/pdf/Annex3_closure_rehabilitation%20.pdf)
591 [%20.pdf](https://ec.europa.eu/environment/waste/mining/pdf/Annex3_closure_rehabilitation%20.pdf)

592 European Commission. 2010. EC Guidance on undertaking new non-energy extractive
593 activities in accordance with Natura 2000 requirements. ISBN 978-92-79-18646-2
594 pp. 144Gold, C., Feurtet-Mazel, A., Coste, M., Boudou, A., 2003. Impacts of Cd and
595 Zn on the development of periphytic diatom communities in artificial streams located
596 along a river pollution gradient. Archives of Environmental Contamination and
597 Toxicology. 44(2):189–197. doi: 10.1007/s00244-002-2024-3.

598 Guasch, H., Atli, G. Bonet, B. Corcoll, N. Leira, M. Serra, A. 2010. Discharge and the
599 response of biofilms to metal exposure in Mediterranean rivers. Hydrobiologia 657:
600 143–157. <https://doi.org/10.1007/s10750-010-0116-z>.

601 Guasch H, Paulsson M, Sabater S. 2002. Effect of copper on algal communities
602 from oligotrophic calcareous streams. J Phycol. 38: 241–243.Ivorra, N., Hettelaar,
603 J., Tubbing, M.H.S., Kraak, S., Sabater, W., Admiraal, W., 1999. Translocation of
604 microbenthic algal assemblages used for *in situ* analysis of metal pollution in rivers
605 Archives of Environmental Contamination and Toxicology. 37: 19-28.
606 <https://doi.org/10.1007/s002449900485>

607 Ivorra, N., Barranguet, C., Jonker, M., Kraak, M.H.S., Admiraal, W., 2002. Metal-induced
608 tolerance in freshwater microbenthic diatom *Gomphonema parvum*. Environ
609 Pollut. 116:147–157

610 Ivorra, N., Bremer, S., Guasch, H., Kraak, M.H.S., Admiraal, W., 2000. Differences in the
611 sensitivity of benthic microalgae to Zn and Cd regarding biofilm development and
612 exposure history. Environ. Toxicol. Chem. 19, 1332–1339.
613 <https://doi.org/10.1002/etc.5620190516>

614 Jain, R.K., Cui, Z., Cindy & Domen, J.K., 2016. Environmental Impacts of Mining,
615 Environmental Impact of Mining and Mineral Processing. 3–157.
616 <https://doi.org/10.1016/B978-0-12-804040-9.00004>

617 Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining
618 chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton.

619 Biochem. und Physiol. der Pflanz. 167, 191–194. <https://doi.org/10.1016/S0015->
620 3796(17)30778-3

621 Johnson, D.B., Hallberg, K.B., 2005. Acid mine drainage remediation options: a review.
622 Sci Tot Environ. 338: 3–14. <https://doi.org/10.1016/j.scitotenv.2004.09.002>

623 Joshi, N., Ngwenya, B. T., & French, C. E. (2012). Enhanced resistance to nanoparticle
624 toxicity is conferred by overproduction of extracellular polymeric substances.
625 Journal of hazardous materials, 241, 363-370.

626 Kumar, P. ,2015. Hydrocomplexity: Addressing water security and emergent
627 environmental risks. Water Resources Research, 51(7), 5827-5838

628 Leal-Alvarado, D.A., Espadas-Gil, F., Sáenz-Carbonell, L., Talavera-May, C.,
629 Santamaría, J.M., 2016. Lead accumulation reduces photosynthesis in the lead
630 hyper-accumulator *Salvinia minima* Baker by affecting the cell membrane and
631 inducing stomatal closure. Aquat. Toxicol. 171, 37–47.
632 <https://doi.org/10.1016/j.aquatox.2015.12.008>

633 Leguay, S., Lavoie, I., Levy, J.L., Fortin, C., 2016. Using biofilms for monitoring metal
634 contamination in lotic ecosystems: The protective effects of hardness and pH on
635 metal bioaccumulation. Environ. Toxicol. Chem. 35, 1489–1501.
636 <https://doi.org/10.1002/etc.3292>

637 Lin, D., Story, S. D., Walker, S. L., Huang, Q., & Cai, P. (2016). Influence of extracellular
638 polymeric substances on the aggregation kinetics of TiO₂ nanoparticles. Water
639 research, 104, 381-388.

640 Liu, J., Tang, J., Wan, J., Wu, C., Graham, B., Kerr, P. G., & Wu, Y. (2017). Functional
641 sustainability of periphytic biofilms in organic matter and Cu²⁺ removal during
642 prolonged exposure to TiO₂ nanoparticles. Journal of hazardous materials.

643 Maher, A., Sadeghi, M., & Moheb, A. (2014). Heavy metal elimination from drinking water
644 using nanofiltration membrane technology and process optimization using response
645 surface methodology. Desalination, 352, 166-173

646 Margalef, R., 1983. Limnología. Ediciones Omega, S.A., Barcelona. 1010p.

647 Miller, R.J., Muller, E.B., Cole, B., Martin, T., Nisbet, R., Bielmyer-Fraser, G.K., Jarvis,
648 T.A., Keller, A.A., Cherr, G., Lenihan, H.S., 2017. Photosynthetic efficiency predicts
649 toxic effects of metal nanomaterials in phytoplankton. Aquat. Toxicol. 183, 85–93.
650 <https://doi.org/10.1016/j.aquatox.2016.12.009>

651 Mohammad, A. W., Teow, Y. H., Ang, W. L., Chung, Y. T., Oatley-Radcliffe, D. L., & Hilal,
652 N. (2015). Nanofiltration membranes review: Recent advances and future
653 prospects. *Desalination*, 356, 226-254

654 Morin, S., Cordonier, A., Lavoie, I., Arini, A., Blanco, S., Becares, E., Coste, M.,
655 Faggiano, L., Laviale, M., Pe, F., Fortin, C., Guasch, H., Sabater, S., 2012.
656 Consistency in diatom response to metal-contaminated environments. In: *Emerging*
657 *and Priority Pollutants in Rivers.*, pp 117-146, [https://doi.org/10.1007/978-3-642-](https://doi.org/10.1007/978-3-642-25722-3)
658 [25722-3](https://doi.org/10.1007/978-3-642-25722-3)

659 Morin, S., Vivas-Nogues, M., Duong, T.T., Boudou, A., Coste, M., Delmas, F., 2007.
660 Dynamics of benthic diatom colonization in a cadmium/zinc-polluted river (Riou-
661 Mort, France). *Fundam. Appl. Limnol. / Arch. für Hydrobiol.* 168, 179–187.
662 <https://doi.org/10.1127/1863-9135/2007/0168-0179>

663 Moussa, D. T., El-Naas, M. H., Nasser, M., & Al-Marri, M. J. (2017). A comprehensive
664 review of electrocoagulation for water treatment: Potentials and challenges. *Journal*
665 *of environmental management*, 186, 24-41

666 Murphy, J.P., Riley, A., 1962. Modified single solution method for the determination of
667 phosphate in natural waters. *Anal. Chim. Acta.* 27:31–36,
668 [https://doi.org/10.1016/S0003-2670\(00\)88444-5](https://doi.org/10.1016/S0003-2670(00)88444-5)

669 Natural Resources of Wales (2016). Abandoned Mine Case Study: Frongoch Lead &
670 Zinc Mine.

671 Oatley-Radcliffe, D. L., Walters, M., Ainscough, T. J., Williams, P. M., Mohammad, A.
672 W., & Hilal, N. (2017). Nanofiltration membranes and processes: A review of
673 research trends over the past decade. *Journal of Water Process Engineering*, 19,
674 164-171

675 Pawlik-Skowronska, B., 2003. When adapted to high zinc concentration the periphytic
676 green alga *Stigeoclonium tenue* produces high amounts of novel phytochelatin –
677 related peptides. *Aquat. Toxicol.* 62, 155-163 Prieto, F., Barrado, E., Vega, M. et al.
678 *Russian Journal of Applied Chemistry* (2001) 74: 1321.
679 <https://doi.org/10.1023/A:1013710413982>

680 Pistocchi, R., Mormile, M. A., Guerrini, F., Isani, G., & Boni, L. (2000). Increased
681 production of extra-and intracellular metal-ligands in phytoplankton exposed to
682 copper and cadmium. *Journal of Applied phycology*, 12(3-5), 469-477.

683 Quigg, A., Chin, W. C., Chen, C. S., Zhang, S., Jiang, Y., Miao, A. J., ... & Santschi, P.

684 H. (2013). Direct and indirect toxic effects of engineered nanoparticles on algae:
 685 role of natural organic matter. *ACS Sustainable Chemistry & Engineering*, 1(7), 686-
 686 702.

687 Reardon J., Foreman J. A., Searcy R. L., 1966. New reactants for the colorimetric
 688 determination of ammonia. *Clin. Chim. Acta.* 14, 203–205.
 689 [https://doi.org/10.1016/0009-8981\(66\)90120-3](https://doi.org/10.1016/0009-8981(66)90120-3).

690 Rose, F.L., Cushing, C.E., 1970. Periphyton: Autoradiography of Zinc-65 Adsorption.
 691 *Am. Assoc. Adv. Sci.* 168, 576–577.

692 Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., Schmitt-Jansen,
 693 M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm
 694 as an interface. *Anal. Bioanal. Chem.* 387, 1425–1434.
 695 <https://doi.org/10.1007/s00216-006-1051-8>

696 Serra, A., 2009. Fate and effects of copper in fluvial ecosystems: the role of periphyton.
 697 PhD Thesis. Girona (Spain), pp. 110–133.

698 Sheng, G. P., Yu, H. Q., & Li, X. Y. (2010). Extracellular polymeric substances (EPS) of
 699 microbial aggregates in biological wastewater treatment systems: a review.
 700 *Biotechnology advances*, 28(6), 882-894.

701 Solomon, F., 2008. Impacts of metals on aquatic ecosystems and human health.
 702 MINING.com.
 703 <http://www.infomine.com/library/publications/docs/Mining.com/Apr2008c.pdf>

704 Tang, J., Wu, Y., Esquivel-Elizondo, S., Sørensen, S. J., & Rittmann, B. E. (2018). How
 705 microbial aggregates protect against nanoparticle toxicity. *Trends in biotechnology*,
 706 36(11), 1171-1182. Tang, J., Zhu, N., Zhu, Y., Kerr, P., & Wu, Y. (2017).
 707 Distinguishing the roles of different extracellular polymeric substance fractions of a
 708 periphytic biofilm in defending against Fe₂O₃ nanoparticle toxicity. *Environmental*
 709 *Science: Nano*, 4(8), 1682-1691.

710 Tang, J., Zhu, N., Zhu, Y., Liu, J., Wu, C., Kerr, P., ... & Lam, P. K. (2017). Responses
 711 of periphyton to Fe₂O₃ nanoparticles: a physiological and ecological basis for
 712 defending nanotoxicity. *Environmental science & technology*, 51(18), 10797-10805.

713 Tlili, A., Bérard, A., Roulier, J.L., Volat, B., Montuelle, B., 2010. PO₄³⁻ dependence of
 714 the tolerance of autotrophic and heterotrophic biofilm communities to copper and
 715 diuron. *Aquatic toxicology* (Amsterdam, Netherlands). 98. 165-77.
 716 [10.1016/j.aquatox.2010.02.008](https://doi.org/10.1016/j.aquatox.2010.02.008).

- Tlili, A., Corcoll, N., Bonet, B., Morin, S., Montuelle, B., Berard, A., Guasch, H., 2011. In situ spatio-temporal changes in pollution-induced community tolerance to zinc in autotrophic and heterotrophic biofilm communities. *Ecotoxicology*, 20 (8), 1823-1839., doi: 10.1007/s10646-011-0721-2
- Tripathi, A., Rawat Ranjan, M., 2015. Heavy Metal Removal from Wastewater Using Low Cost Adsorbents. *J. Bioremediation Biodegrad.* 06. <https://doi.org/10.4172/2155-6199.1000315>
- USEPA (United States Environmental Protection Agency), 2014. Reference Guide to Treatment Technologies for Mining-Influenced Water. EPA 542-R-14-001.
- Wu, Y. (2016). *Periphyton: functions and application in environmental remediation*. Elsevier.
- Ylla, I., Borrego, C., Romani, A.M., Sabater, S., 2009. Availability of glucose and light modulates the structure and function of a microbial biofilm. *FEMS Microbiol. Ecol.* 69, 27–42. <https://doi.org/10.1111/j.1574-6941.2009.00689.x>
- Younger, P.L., Amezaga, J., Baresel, C., Destouni, G., Gren, I., Hannerz, F., 2003. Mining Impacts on the Fresh Water Environment: Technical and Managerial Guidelines for Catchment Scale Management. *Environ* 23:s2-s80. <https://doi.org/10.1007/s10230-004-0028-0DO> .
- Zhu, N., Wu, Y., Tang, J., Duan, P., Yao, L., Rene, E. R. & Dionysiou, D. D. (2018). A new concept of promoting nitrate reduction in surface waters: simultaneous supplement of denitrifiers, electron donor pool, and electron mediators. *Environmental science & technology*, 52(15), 8617-8626
- Zhu, N., Wang, S., Tang, C., Duan, P., Yao, L., Tang, J. & Wu, Y. (2019). Protection mechanisms of periphytic biofilm to photocatalytic nanoparticle exposure. *Environmental science & technology*, 53(3), 1585-1594.